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Basic fibroblast growth factor can mediate the early inductive events in renal development

(FGF2/c-*met*/kidney/*WT1*)

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ABSTRACT **The earliest characterized events during induction of tubulogenesis in renal anlage include the condensation or compaction of metanephrogenic mesenchyme with the concurrent upregulation of *WT1*, the gene encoding the Wilms tumor transcriptional activator/suppressor. We report that basic fibroblast growth factor (FGF2) can mimic the early effects of an inductor tissue by promoting the condensation of mesenchyme and inhibiting the tissue degeneration associated with the absence of an inductor tissue. By *in situ* hybridization, FGF2 was also found to mediate the transcriptional activation of *WT1* and of the hepatocyte growth factor receptor gene, c-*met*. Although FGF2 can induce these early events of renal tubulogenesis, it cannot promote the epithelial conversion associated with tubule formation in metanephrogenic mesenchyme. For this, an undefined factor(s) from pituitary extract in combination with FGF2 can cause tubule formation in uninduced mesenchyme. These findings support the concept that induction in kidney is a multiphasic process that is mediated by more than a single comprehensive inductive factor and that soluble molecules can mimic these inductive activities in isolated uninduced metanephrogenic mesenchyme.**

Stem-cell commitment during embryogenesis and organogenesis is often mediated by the interaction of an inductor tissue with a targeted undifferentiated cell population. During gastrulation, interactions between the animal and vegetal hemispheres—i.e., presumptive ectoderm and endoderm—of the *Xenopus* blastula result in the formation of mesoderm and its derivatives such as skeletal muscle (1). Similarly, bidirectional signaling between mesenchymal and epithelial rudiments directs growth, morphogenesis, and tissue organization later in the development of most parenchymal tissues (2). The molecular bases for these interactions remain largely undefined; however, recent studies in *Xenopus* have demonstrated that diffusible factors can mimic the effects of the inductor tissue in the formation of mesodermally derived tissues, notably in the induction of skeletal muscle, and members of the fibroblast growth factor (FGF) family have been implicated (3). Similarly in the chicken, basic FGF (FGF2) can replace the inductive effect of the ectodermal apical ridge in the maintenance and elongation of the underlying mesoderm to allow limb development (4).

Renal development is characterized by a reciprocal interaction between an epithelial ureteric bud and the metanephrogenic mesenchyme, both of mesodermal origin (for review, see ref. 5). As a result, the mesenchyme is converted to a primitive epithelium that subsequently forms the diverse structures of the nephron. While a multitude of growth/differentiation-inducing factors, both diffusible and nondiffusible, have been identified in renal rudiments (6, 7), none has been shown individually to induce tubulogenesis in metanephrogenic mesenchyme. This suggests that a combination of such factors may be required for induction. Indeed this appears to be the case, since tubulogenesis can occur in culture when uninduced mesenchyme is treated with a series of defined soluble components, an insoluble matrix, and growth factor-enriched pituitary extract (8). Furthermore, suppression of certain growth regulatory molecules—e.g., the receptor Ret (9), the transcription factor WT1 (10), nerve growth factor receptor (11), insulin-like growth factors I and II (12), transforming growth factor α (13), or hepatocyte growth factor (HGF) (14)—can block renal development, potentially implicating these several factors in the induction process. However, with the exception of WT1, all have been implicated through studies of intact rudiments, which cannot distinguish direct interference with inductive signaling from indirect effects such as growth suppression of the inductor bud. Accordingly, we have evaluated the effects of growth/differentiation factors directly on isolated uninduced metanephrogenic mesenchyme and now report that FGF2 can mediate the early events of renal differentiation.

METHODS

Tissues. Timed pregnant F344 rats were euthanized at gestational day 13 (gd13; the day a spermatic plug was observed was designated as gd0), and embryonic kidneys were surgically excised in phosphate-buffered saline. Metanephrogenic mesenchymes were then isolated from the buds and cultured as described (8). Only mesenchymes from rudiments in which the bud had just begun its primary branching were used, since at this stage it has not been induced to form tubules.

Purification of Condensing Activity. Bovine pituitary extract was prepared for induction studies as described (15) and then fractionated by ammonium sulfate precipitation. Inductive activity was precipitable between 25% and 65% saturation. Pellets were resuspended and dialyzed in 50 mM Tris·HCl (pH 7.5) and then acidified with 20% glacial acetic acid to pH 4.5. Neutralized supernatants were applied to a 15-ml heparin-Sepharose column prepared as recommended by the manufacturer, and proteins were eluted with stepwise increases in NaCl. For heparin-affinity HPLC, dialyzed fractions were pumped onto tandem Bio-Rad heparin-affinity Econo columns at 0.5 M NaCl and eluted with stepwise increases in NaCl. Fractions were concentrated and desalted in Ultrafree-CL microconcentrators (Millipore). Biological activity was determined by the ability of a fraction to induce the condensation of isolated gd13 metanephrogenic mesenchymes, which occurs within 24 hr of treatment.

Immunoblotting. Immunoblotting was performed as described by Gallagher (16). Proteins separated in an SDS/4–20% polyacrylamide gel were electroblotted to a 0.45-μm nitrocellulose filter. Immunoblots were probed either with a

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Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; gd*n*, gestational day *n*.

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monoclonal antibody to FGF2 (Upstate Biotechnology) or a polyclonal antibody to acidic FGF (FGF1) (Sigma), each at 1:1000 in Tris-buffered saline containing Tween 20, and visualized with a horseradish peroxidase-labeled secondary antibody and diaminobenzidine.

***In Situ* Hybridization.** Separated mesenchymes were cultured on Nuclepore filters and fixed in buffered 4% paraformaldehyde, and the filters were embedded in paraffin perpendicular to the cutting surface. Labeling of probes and *in situ* hybridization were performed according to Wilkinson and Green (17). A 325-bp fragment of human cDNA for WT1 was cloned into the pCR-Script SK(+) (Stratagene) vector. This probe includes most of exons 1–3 and recognizes mouse and rat RNA by *in situ* hybridization. For the HGF receptor gene (the c-*met* protooncogene), a 990-bp fragment that included the 3′ end of the extracellular domain, the transmembrane domain, and the 5′ end of the cytoplasmic domain generated from gd16 embryonic rat kidney RNA was cloned into pCR-Script SK(+). The insert was sequenced and showed high homology with the same region of mouse c-*met* sequence.

RESULTS

Purification of a Condensing Factor in Pituitary Extract. The morphogenesis of explanted metanephrogenic mesenchyme from the mouse involves a series of well-defined events, which are reproducible with a variety of alternative inductor tissues (most notably embryonic brain and spinal cord). Upon recombination with an inductor, the mesenchyme condenses within 24 hr and forms tubules after an additional two to several days (18). In the absence of an inductor, the mesenchyme spreads in a monolayer on the substratum. In earlier studies, we determined that several growth factors expressed in kidney were incapable of mimicking the inductive activity either individually or in combination (8). Similar findings were reported for mouse mesenchymes (19). Therefore, we initiated efforts to purify the inducing activity we had demonstrated (8) from homogenates of pituitary. Studies utilized explanted gd13 rat metanephrogenic mesenchyme, which is comparable developmentally to uninduced gd10.5 mouse anlage. The initial endpoint was condensation of the mesenchymes, the earliest described morphogenic event in tubulogenesis (18). Activity capable of condensing mesenchyme and causing tubule formation was retained in ammonium sulfate precipitate (25–65% saturation) and in acetic acid-treated supernatant from pituitary extract. Since heparin has been shown to severely limit nephron development in cultured renal rudiments (20) and completely blocks tubule formation in cultured mesenchymes treated with pituitary extract (data not shown), the ammonium sulfate-precipitated, acid-treated fraction of pituitary extract was applied to a heparin-Sepharose column. Condensing activity was retained in the 0.8–2 M NaCl eluate. Furthermore, when dialyzed 0.8–2 M NaCl eluate was applied to two tandem heparin-affinity HPLC columns, condensing activity (2-ml fractions from 52 to 56 ± 2 min) was separated from the majority of protein (Fig. 1) and eluted at 1.25–1.5 M NaCl. These fractions, however, were unable to promote the condensed mesenchyme to form tubules.

FGF2 Is the Condensing Factor. Proteins in HPLC fractions were separated by SDS/4–20% PAGE (Fig. 2), and silver staining revealed a single intense band at ≈18 kDa in those fractions with condensing activity. In studies of FGF2 in pituitary (21), a single protein immunoreactive with antiserum to FGF2 was eluted at 1.4 M NaCl from a heparin-affinity matrix. To determine whether the condensing or compacting factor was FGF2, separated fractions were immunoblotted (Fig. 3) with antibodies specific for FGF1 or FGF2. HPLC fractions with condensing activity showed strong signals with the antibody for FGF2, but not with antibody for FGF1.

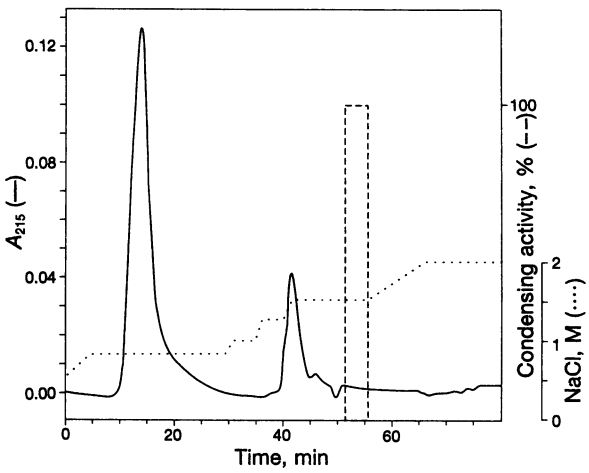

FIG. 1. HPLC separation of condensing activity for metanephrogenic mesenchyme from pituitary extract. Separations were performed on two 5-ml Bio-Rad heparin-affinity columns with stepwise NaCl gradient elution at a flow rate of 1 ml/min. Condensing activity (- - - -) was eluted in fractions from 52 to 56 ± 2 min.

To verify this observation directly, freshly isolated mesenchymes from gd13 rat renal rudiments were explanted to a defined medium containing a commercial preparation of recombinant bovine FGF2. In the presence of FGF2 (50–200 ng/ml), three-dimensional condensates (Fig. 4*a*) appeared and maintained their compacted form with increasing mass over a 2-week period during which there were no obvious indications of tubule formation. In the absence of FGF2, tissue masses rapidly deteriorated, and by 3 days in culture, the few remaining cells had spread in monolayer (Fig. 4*b*).

FGF2 Promotes the Upregulation of *WT1* and c-*met* Expression. Upregulation of *WT1* expression during kidney development is one of the earliest molecular events immediately following the interaction between mesenchyme and inductor tissue (22). High expression is observed specifically in condensing mesenchyme adjacent to branch termini of the ureteric bud, while expression is very weak in uninduced mesenchyme and absent in ureteric bud. To evaluate *WT1* expression in mesenchymal condensates, FGF2-treated cultures of uninduced mesenchyme were subjected to *in situ* hybridization 1 and 3 days after explanation. Hybridization in tissue sections with ^{35}S-labeled antisense probes showed that expression was induced within 24 hr in areas where the first morphological signs of condensation could be detected (Fig. 5 *a* and *b*). This expression was more dramatic after 3 days of treatment, when the condensations had increased in size and density (Fig. 5 *c* and *d*). Similarly, c-*met* expression has been documented in embryonic mouse kidney, although initial studies associated its

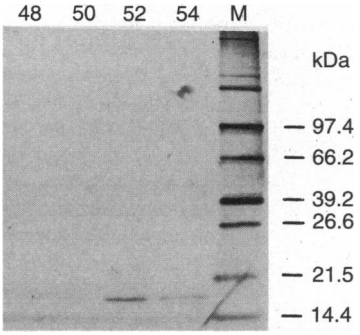

FIG. 2. Silver-stained SDS/4–20% polyacrylamide gel of separated proteins in heparin-affinity HPLC fractions. A single 18-kDa band was observed in fractions containing condensing activity. Lane M, protein size markers.

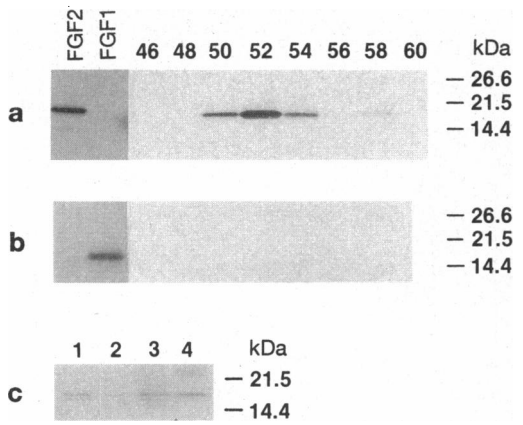

FIG. 3. Immunoblot of HPLC fractions or tissues with antisera to FGF. (*a* and *b*) HPLC fractions were electrophoresed in SDS/4–20% polyacrylamide gels and immunoblotted for FGF2 (*a*) or FGF1 (*a*). (*c*) Inductor tissues were probed for FGF2. Lanes: 1, gd15 rat kidney; 2, gd13 spinal cord; 3, gd13 brain; 4, pituitary extract.

expression with the primitive epithelia (23). Most recently, expression was demonstrated in induced immortalized mesenchymal cells derived from early metanephros (24). By *in situ* hybridization in sections from gd13–19 rat kidneys, we have detected c-*met* transcripts not only in the tips of the ureteric bud, as was shown by Sonnenberg *et al.* (23), but also in the induced condensing mesenchyme surrounding the tips (I.K., unpublished work). In our explant cultures of mesenchyme, FGF2 treatment resulted in the elevated expression of c-*met* within 24 hr (Fig. 5 *e–h*). Like the *WT1* transcripts, c-*met* transcripts appeared as soon as the first condensates began to form and persisted in these condensed areas but not in adjacent loosely packed mesenchyme. Transcripts for c-*met* and *WT1* were not detected in the monolayers that formed from untreated explants after 3 days. Sense probes for both sequences also failed to hybridize to the sections.

FGF2 Is Necessary but Not Sufficient for Tubulogenesis in Metanephrogenic Mesenchyme. Studies of heterologous inductive tissues showed that certain tissues were capable of inducing only condensation and not tubule formation (18), which suggests the involvement of multiple factors in tubulogenesis. In the current studies, purification of the condensation-inducing factor—i.e., FGF2—resulted in the loss of tubule-inducing activity from any heparin-affinity HPLC fractions of pituitary extract. To examine whether these activities are distinct, we cultured mesenchymes with FGF2 (100 ng/ml) and fractions that had been depleted of FGF2 and eluted from heparin-Sepharose columns with increasing NaCl concentrations. For gd13 mesenchymes cultured with FGF2, tissue masses formed condensates within 24 hr as described above. In cultures treated for 6 days with FGF2 plus the non-heparin-binding fraction from a heparin-Sepharose column, tubule formation was generally not observed (Fig. 6*a*). A fraction eluted from heparin-Sepharose with 0.5 M NaCl contained no tubule-inducing activity in the absence of FGF2 (Fig. 6*b*). However, when explants were treated with FGF2 and the 0.5 M NaCl eluant, extensive tubule formation occurred, beginning within 3 days of treatment and reaching a maximal level by day 6 (Fig. 6*c*). Histologic examination of the explants revealed numerous blastemal and tubular elements (Fig. 6*d*). Tubulogenesis therefore may require the presence of at least two distinct factors: an initial condensing activity, which is mimicked by FGF2, and an additional heparin-binding factor that in combination provides a tubule-inducing activity.

FGF2 Is Produced in Embryonic Kidney and Potent Inductor Tissues. While FGF2 has been purified from adult bovine kidney (25) and brain (26), its formation during early renal or neurogenic development has not been established. Accordingly, gd15 renal rudiments from rats (the earliest day from which sufficient biological material is available for gel analysis) or one of two potent inductor tissues, gd13 embryonic brain or spinal cord, were solubilized and proteins were immunoblotted for FGF2. A doublet around 18 kDa was observed for each tissue probed with antibody to FGF2 (Fig. 3*c*). Pituitary extract yielded an 18-kDa band and a larger reactive polypeptide of 24 kDa. Thus, FGF2 immunoreactive material is produced in the embryonic kidney and in nonrenal inductor tissues.

DISCUSSION

The current experimental evidence demonstrates that FGF2 can mimic the effects of established inductor tissues for metanephrogenic mesenchyme and cause the upregulation of two genes that appear to be required for renal morphogenesis—i.e., *WT1* and c-*met*. *WT1* expression during early kidney development in human and mouse rudiments occurs predominantly in condensing mesenchyme and not in the ureteric bud (22). When renal vesicles appear following epithelial conversion, *WT1* expression is observed predominantly in those epithelial cells destined to become podocytes in the glomerulus. As a marker for developing kidney, its expression in mesenchymal cells is specifically associated with induction, and its upregulation in FGF2-treated mesenchyme, therefore, indicates that the induction process has been initiated. For c-*met*, expression has been postulated to participate in the epithelial conversion process itself, since transcripts have been localized to primitive epithelia (23) and since coexpression of c-*met*, which encodes the HGF receptor, and the HGF gene apparently generates epithelial markers in mouse NIH 3T3 fibroblasts (27). However, recent demonstrations that an immortalized cell from metanephrogenic mesenchyme can be induced to express c-*met* without undergoing epithelial conversion (24) and that mesenchymal tumors often coexpress the HGF gene and c-*met* (28) suggest that these events are not necessarily linked. They also show that c-*met* expression is often associated with mesenchymal cells and, in the case of the immortalized cells, is specifically a concomitant of induction.

By immunoblotting, proteins immunoreactive with antibody specific for FGF2 were found in gd15 rat kidney and in two potent inductor tissues, gd13 spinal cord and brain. Adult

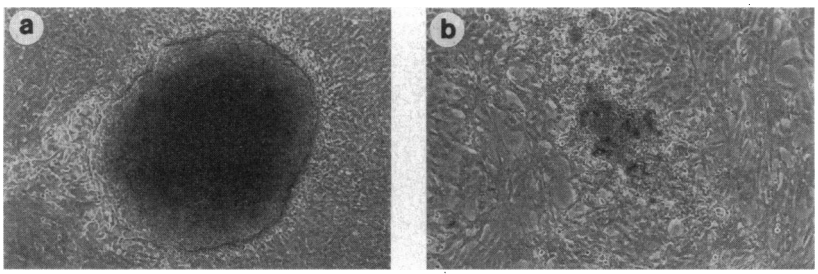

FIG. 4. Isolated gd13 rat metanephrogenic mesenchyme treated with (*a*) or without (*b*) FGF2 (100 ng/ml) for 3 days in culture. Three-dimensional condensates formed only in the presence of FGF2. (×50.)

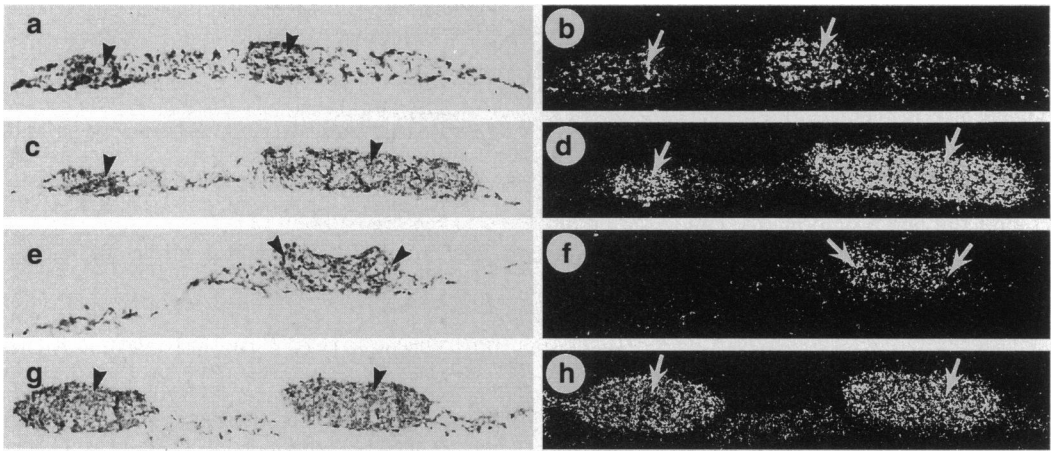

FIG. 5. Expression of *WT1* and c-*met* mRNA in cultured gd13 metanephrogenic mesenchyme treated with FGF2. Bright field (*Left*) and dark field (*Right*) photomicrographs show tissue sections probed with *WT1* antisense sequence after 1 day (*a* and *b*) or 3 days (*c* and *d*) in culture or with c-*met* antisense sequence after 1 day (*e* and *f*) or 3 days (*g* and *h*). Expression is localized to the condensed areas (arrowheads), whereas the surrounding loose mesenchyme is negative. (×65.)

bovine kidney has been used in the past for purification purposes as an enriched source of both FGF2 (25) and FGF1 (29). In the developing metanephros, an undefined heparin-binding protein of 16–20 kDa with angiogenic activity has been identified in mouse renal rudiments at gd11, which is the developmental equivalent of gd13 in rat, and represents a preinduction state in renal organogenesis (30). Coincident with the expression of this presumed FGF family member are specific FGF receptor (FGFR) isoforms, which have been localized histiotypically in the metanephros. FGFR1 occurs in a variety of mesenchymal cells during development (31) and exhibits a high affinity for FGF2 (32). FGFR1 (*flg*) transcripts have been localized to the metanephrogenic mesenchyme (31), whereas transcripts for FGFR2 (keratinocyte growth factor receptor isoform but not *bek*) are found preferentially in the ureteric bud (33). Thus, the presence of a protein reactive with an antibody to FGF2 in renal primordia and inductor tissues and the localization of an appropriate receptor in mesenchyme during the early stages of renal organogenesis are consistent with their participation in the induction process. Obviously, the involvement *in vivo* of other family members cannot be ruled out. Several forms of FGF have been detected in the developing *Xenopus* embryo at the time of gastrulation (34), and one or more may be responsible for induction of mesoderm.

Saxén (5) describes kidney tubule induction as a permissive event—i.e., one in which the inductor stimulates a predetermined population of metanephrogenic mesenchyme to form tubules as the only option in development other than remaining as stroma. Such an induction could be achieved either through an active stimulation of the developmental process or by a passive indirect mechanism involving inhibition of apoptosis in cells destined to form tubules. In the absence of an inductive tissue as shown here and by others (35), metanephrogenic mesenchyme rapidly degenerates apparently by apoptosis, but epidermal growth factor (EGF) can significantly inhibit the DNA degradation associated with this process. Inhibition alone, however, is not sufficient to permit tubulogenesis, since none was observed in EGF-treated cultures (19). We found that EGF by itself could maintain a monolayer of mesenchymal cells but had no effect on the ability of the mesenchymal masses to retain their compacted three-dimensional configuration (unpublished observation). The addition of FGF2 was required for compaction and subsequently for tubulogenesis. While this suggests that EGF may function passively by inhibiting programed cell death and that FGF2 may actively promote differentiation, it has been shown at least for vascular endothelial cells that FGF deprivation can result in apoptosis (36), so it is possible that both growth factors behave as survival factors. An examination of mouse

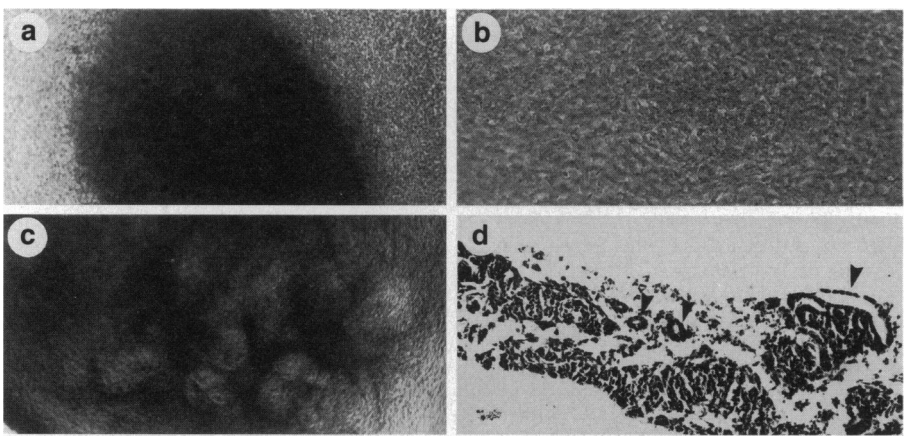

FIG. 6. Induction of tubule formation in gd13 rat metanephrogenic mesenchyme. (*a–c*) Isolated mesenchymes were treated for 6 days with FGF2 (100 ng/ml) plus the non-heparin-binding fraction from pituitary extract (*a*), 0.5 M NaCl eluate from a heparin-Sepharose column (*b*), or FGF2 plus 0.5 M NaCl eluate (*c*). (*d*) Hematoxylin- and eosin-stained tissue section of explanted mesenchymes treated as in *c*. Tubule formation (arrowheads) occurred in several areas in the explant and only when both FGF2 and a second heparin-binding component from pituitary extract were present. (*a–c*, ×50; *d*, ×65.)

renal rudiments bearing an inactivating germline mutation in *WT1* revealed massive apoptosis of the blastemal mesenchymal cells, subsequent total degeneration of this cell population, and the complete absence of kidney formation (10). Even recombinations of isolated mesenchymes with normal inductor tissues failed to promote growth or tubule formation. Thus, normal *WT1* appears to function in a manner similar to that described here for FGF by preventing the degeneration of the mesenchymal component and promoting the condensation of mesenchymal cells.

Although we did not examine the basis for the condensation of mesenchyme, syndecan and Wnt-4 have been postulated to function in this capacity. The membrane-bound glycoprotein syndecan is immunolocalized to induced aggregating mesenchymal cells and is later lost from the epithelial structures in the embryonic kidney (37). While its presence may enhance responsiveness to FGF2 by participating in the formation of a high-affinity receptor for FGF2 (38), it has also been shown to regulate cell adhesion (39) and may therefore function in morphogenic events as well. Alternatively, recent examination of mouse embryos carrying a homozygous null allele for *Wnt-4* (40) has demonstrated an inability of metanephrogenic mesenchyme to undergo compaction upon interaction with normally branching ureteric bud. Since Wnt-1 can modulate cell adhesion (41) and the expression of E-cadherin (42), the secreted Wnt-4 protein may function either directly or indirectly in mesenchymal condensation by regulating cell adhesion. In any event, an evaluation of the effect of FGF2 on the expression of these putative adhesion factors may provide insight into the molecular events responsible for condensation.

The involvement of FGF in renal development may explain one of the well-characterized phenomena of the cultured renal anlage—i.e., the potency of neurogenic tissues as inductors. Their effectiveness may be attributable, at least in part, to the fact that they are exceptional sources of FGF in embryonic (as shown here), fetal (43), and (25, 43) adult tissues. Although Lombard and Grobstein (44) reported that the ability of brain to serve as an inductor of kidney tubule formation diminished with maturation and that the adult brain was incapable of inducing tubulogenesis, it was also observed that both embryonic and adult brain tissue could induce the formation of condensates of mesenchyme (18) similar to those generated here with FGF2. In all likelihood, a member of the FGF family is responsible for the condensing activity elicited by the neurogenic inductor tissues. Furthermore, the dichotomy of responses described in these earlier studies reinforces the multistep concept of the induction process and supports our contention that no single factor can induce tubule formation; instead, at least two and perhaps several more are required.

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